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(54) **Title:** REPRESSOR OF ESTROGEN RECEPTOR ACTIVITY

(57) **Abstract**

The action of nuclear hormone receptors is tripartite, involving the receptor, its ligands, and its coregulator proteins. The estrogen receptor (ER), a member of this superfamily, is a hormone–activated transcription factor that mediates the stimulatory effects of estrogens and the inhibitory effects of antiestrogens like tamoxifen in breast cancer and other estrogen target cells. To understand how antiestrogens and dominant negative ERs suppress ER activity, a dominant negative ER was used as bait in two–hybrid screening assays from which a clone from breast cancer cells was isolated that potentiates the inhibitory activities of dominant negative ERs and in antiestrogen–liganded ER. At higher concentrations, it also represses the transcriptional activity of the estradiol–liganded ER, while having no effect on other nuclear hormone receptors. This clone, denoted REA for "repressor of estrogen receptor activity", encodes a 37 kDa protein that is the first ER–selective coregulator to be identified. Its competitive reversal of steroid receptor coactivator (SRC–1) enhancement of ER activity and its direct interaction with liganded ER indicate that it plays an important role in determining the sensitivity of estrogen target cells, including breast cancer cells, to antiestrogens and estrogens.

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REPRESSOR OF ESTROGEN RECEPTOR ACTIVITY

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from United States Provisional Patent Application Serial No. 60/107,046 filed November 4, 1998.

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BACKGROUND OF THE INVENTION

The steroid hormone receptors constitute a class of proteins to which specific steroids bind and which function as transcription factors that regulate various genes that affect growth, development and homeostasis. The steroid hormone receptors include the glucocorticoid receptor, progesterone receptor, estrogen receptor (ER), mineralocorticoid receptor, androgen receptor as well as receptors for thyroid hormone, retinoic acid, vitamin D and other compounds. Steroid hormones enter cells by passive diffusion and interact with their respective receptors in the cytoplasm or nucleus. The binding of hormone to receptor is said to activate the receptor. Activated receptors are able to bind to cognate recognition DNA sequences associated with target genes affected by the hormone and regulate gene transcription. Changes in mRNA synthesis result in altered expression levels of regulated genes, which

ultimately determine the functional response of target tissue to steroid hormones. The basic regulation system is modulated and fine-tuned by a complex array of agonists, antagonists and co-regulator proteins including co-activators and co-repressors, some of which interact with several different receptors and some of which are specific to a single receptor. For a general review of steroid hormone receptors and their regulation, see, e.g., Katzenellenbogen, J.A., et al. (1996) *Mol. Endocrinol.* **10**:119-131; Shibata, H., et al. (1997) *Recent Prog. Hormone Res.* **52**:141-1651 Mauglesdorf, D.J., et al. (1995) *Cell* **83**:835-839; Horwitz, K.B., et al. (1996) *Mol. Endocrinol.* **10**:1167-1177.

The estrogen receptor (ER) stimulates the expression of specific estrogen-regulated genes. The ER shares many of the structural and functional domain organizational features of other steroid hormone receptors. Certain estrogen-regulated genes have a role in the growth and progression of breast cancers. Therapeutic benefits have been observed by treatment with antiestrogens such as Tamoxifen. Antiestrogens compete with estrogens for binding to the ER but fail to activate the ER, and they are widely used in breast cancer treatment. For reviews, see, e.g. Katzenellenbogen, B., et al. (1997) *Breast Cancer Res. Treat.* **44**:23-28; Dickson, R.B., et al. (1987) *Endocr. Res.* **8**:29-43; Santen, R., et al. (1990) *Endocr. Rev.* **11**:578-610; Ince, B.A., et al. (1993) *J. Biol. Chem.* **268**:14026-14032.

The activation function 2 (AF-2) region of the ER is known to be involved in interaction of ER with other protein factors [Shibata, H. et al. (1997)]. The AF-2 region of ER is also known to undergo a conformation shift in an ER-antiestrogen complex [Brzowski, A.M., et al. (1997) *Nature* **389**:753-758]. A dominant negative ER mutant is known to have a point mutation, L540Q, lying in the AF-2 region [Ince, B.A., et al. (1993); Ince, B.A. et al. (1995) *Endocrinology* **136**:3194-3199; Schodin, D.J., et al. (1995) *J. Biol. Chem.* **270**:31163-31171]. The dominant negative ER mutants lack transcriptional activity and suppress the activity of wild-type ER when they are co-expressed in the same cells.

The protein SRC-1 (steroid receptor co-activator 1) enhances the transcriptional activity of several steroid hormone receptors, including the ER [Shibata, H., et al (1997)]. In the

presence of SRC-1, estradiol-mediated transcription is enhanced 4-5-fold [McInerney, E.M., et al. (1996) *Proc. Natl. Acad. Sci. USA* **93**:10069-10073].

Co-repressors have been identified for nuclear non-steroid hormone receptors [see Shibata, H., et al. (1997)]. Indirect evidence of the existence of a co-repressor of the ER was reported by Lee, H.S., et al (1996) *J. Biol. Chem.***271**:25727-25730. The evidence suggested a protein that interacts with the ligand binding domain of the ER in the absence of hormone.

A protein named B-cell receptor-associated protein (BAP-37) has been identified in B cells [Tereshima, M., et al. (1995) *EMBO J.* **13**:3782-3792; Kim, K-M., et al. (1994) *EMBO J.* **13**:3793-3800]. BAP-37 was shown to be present in cells and tissues other than B-cells. Although sequence data has been reported, the function of the protein has not been characterized. During analysis of the repressor of estrogen receptor activity (REA) described herein, the nucleotide sequences encoding REA and BAP-37 were found to be nearly identical.

SUMMARY OF THE INVENTION

The invention described herein results from experimental evidence demonstrating the existence of a novel co-regulator of the estrogen receptor (ER). The co-regulator, denoted REA for "repressor of estrogen receptor activity" is a 37 kDa protein. The REA is specific for the ER, in contrast with other known co-repressors that exert effects on many hormone receptors. The REA is the first ER-selective co-regulator to be identified.

The activity of REA is primarily manifested by its ability to potentiate the activity of known antiestrogen compounds. It is known that ER in the presence of an estrogen acts to increase transcription of various genes in breast and endometrial cells. Further, it is known that estrogen acts to promote the growth and progression of breast cancers. Antiestrogen compounds, such as Tamoxifen and Raloxifene have been shown to be effective in reducing growth and proliferation of breast cancer cells. In addition, for patients having a genetic susceptibility to breast cancer, Tamoxifen has been shown to prevent or delay the onset of

breast cancer. (Altman, L.K. *New York Times*, April 7, 1998, p.A1.) Nevertheless, at effective dosages, Tamoxifen and other antiestrogens have undesirable side-effects.

In the presence of REA, the dose of antiestrogen required to produce half-maximal repression has been shown to be reduced as much as 50-fold. Therefore, the combination of REA with an antiestrogen compound can result in greater repression and provide therapeutic efficacy at a much lower dose of the drug than is currently employed. In addition, the range of therapeutic indications for Tamoxifen and other antiestrogens can be enlarged to include such conditions as endometriosis, by extending the efficacy of antiestrogen to lower and safer doses.

The REA has now been purified. Recombinant REA modified by addition of an oligo-histidine was purified on a nickel column to yield protein estimated at 95% purity by SDS-PAGE. Antibodies have been prepared against purified REA. Antibodies to REA, either mono- or polyclonal, prepared by known methods, can be used to provide a sensitive and specific assay for REA levels in various tissues. Polyclonal rabbit anti-REA antibodies have been prepared. The antibodies have been used to detect REA on Western immunoblots. Fluorescence labeled antibodies can be used for immuno-staining. Monoclonal antibodies to REA are under preparation. Polyclonal and monoclonal antibodies are useful for quantitative and qualitative assays for REA. Numerous antibody-based assays have been developed, as is well-known in the art, including, but not limited to ELISA and RIA-type assays. Immunoassays for measuring REA are useful for diagnosis and therapy. Individual patient variation in REA level can be measured using an immunoassay to evaluate the likelihood that a given tumor will be responsive to antiestrogen, and, if responsive, to determine the dosage required to be optimally effective. Similarly, a patient's REA level can be assayed to evaluate feasibility and dose of antiestrogen for treatment of endometriosis.

The REA can be used as a therapeutic agent for treatment of breast cancer and other diseases associated with overactivity of ER. In particular, REA can be specifically incorporated in target cells by application of known techniques of gene therapy. The DNA encoding REA can be combined with one of several known mammary cell-specific promoters

or milk protein-related promoters, such that REA is expressed specifically in mammary cells. Transfection of target cells *in vivo* can be accomplished by any of the known vectors, including but not limited to, modified vaccinia and modified adenovirus vectors. Transfected tumor cells expressing REA are thereby rendered more sensitive to antiestrogen therapeutic compounds. In addition, for those patients diagnosed with increased genetic risk of breast cancer, for example, by diagnostic tests to reveal the presence of altered BRCA I or BRCA II gene, gene therapy to increase expression of REA in breast cells can enhance the known preventive activity of antiestrogen compounds such as Tamoxifen.

REA can also be used in a variety of assays to test compounds for estrogen or antiestrogen activity. An *in vitro* assay is based on the ligand-dependent binding of REA with ER. The binding of REA to ER can be measured if either one of the two or both are labeled, in the presence of a putative estrogen or antiestrogen, compared with the binding of known estrogen or antiestrogen. In this *in vitro* assay, antiestrogens promote a stronger interaction between REA and ER than do estrogens. A cell-based assay employs cells in culture transfected with DNA encoding REA together with a reporter gene whose transcription is under control of an ER-regulated reporter. The presence of an estrogen can result in increased levels of reporter gene activity while the presence of an antiestrogen results in reduced activity, relative to controls. This assay can be used to determine the degree to which the inhibitory potency of antiestrogens is enhanced in the presence of REA, and thereby to characterize the suppressive effectiveness of different antiestrogens. In addition, the binding of REA to ER is promoted by an antiestrogen but the binding of SRC-1 to ER is not. A protein-protein binding assay, using labeled REA and labeled SRC-1 can be used to evaluate the effect of a candidate antiestrogen compound. The compound is identified as an antiestrogen if it increases the binding of REA, but not of SRC-1, to ER. Using any of the above-described assay systems, large numbers of compounds, including those generated by combinational chemistries, can be screened for estrogen or antiestrogen activity.

A technique for mapping the domains of REA which interact with receptor and are required for repression has been developed. N-terminal and C-terminal deletion mapping of REA indicates that repression depends on two sequences that lie between amino acids 150 and

174, and amino acids 19 and 49. Both regions appear to be required for repression. Further characterization of functional domains of REA can provide insight into the nature of the interactions and aid in the design of small molecules that modify repressor activity.

The invention therefore includes a method for therapy of a disease associated with overactivity of ER, by providing the affected cells with REA combined with an antiestrogen. In particular the REA can be provided within the affected cells by transforming the cells with a DNA encoding REA combined with a promoter specific for expression in the affected cells.

The invention also provides methods for testing a compound to determine whether or not it has estrogen or antiestrogen activity. An *in vitro* binding assay is provided, as well as a cell-based assay, using cells co-transformed with a gene encoding REA and a reporter gene, the latter being under transcription control of the ER.

The invention provides purified REA, antibody to REA, expression vectors for synthesis of REA in cells transformed by the vector, and labeled REA having an indicator moiety attached thereto or incorporated in its structure. The invention further provides for measuring REA using an immunoassay.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a diagrammatic representation of segments of human REA, shown as horizontal bars, together with the repressive activity associated with each segment. The N-terminal and C-terminal amino acid numbers of each segment are given at the left and right ends, respectively, of each bar.

Fig. 2 shows reporter gene activity for an ER-regulated gene in cultured cells. CAT is chloramphenicol acetyl transferase. E_2 is estradiol.

In Fig. 2A, relative CAT activity calculated as percent of CAT expressed in response to E_2 stimulation (vertical axis) is graphed as a function of increasing level of ERL540Q

(dominant negative ER receptor) on the horizontal axis in the presence (dashed line) or absence (solid line) of 10 ng of REA.

In Fig. 2B, relative CAT activity is plotted as a function of TOT (Trans-hydroxy tamoxifen) concentration in the presence (dashed line) and absence (solid line) of REA.

In Fig. 2C, relative CAT activity is plotted as a function of ICI 182,780 concentration in the presence (dashed line) or absence (solid line) of REA.

In Fig. 2D, relative CAT activity with progesterone receptor is plotted as a function of RU486 concentration in the presence (dashed line) or absence (solid line) of REA.

Fig. 3 is data in bar graph form of reporter gene activity (vertical axis, each panel) regulated by various receptors in the presence of indicated levels of REA (horizontal axis, each panel). The measured activity is chloramphenicol acetyl transferase (CAT).

In Fig. 3A, ERα is estrogen receptor α. The reporter construct is regulated by an estrogen response element (ERE).

In Fig. 3B, ERβ is estrogen receptor β. The reporter construct is that of Fig. 3A.

In Fig. 3C, PR is progesterone receptor. The reporter construct is regulated by mouse mammary tumor virus (MMTV) promoter.

In Fig. 3D, RAR is retinoic acid receptor. The reporter construct is regulated by SV-DR5, a RAR-responsive promoter.

In Fig. 3E, VP16 is an unrelated viral protein (viral protein 16) designated Gal4-VP-16 which regulates a promoter having 5 copies of a Gal4 upstream activating sequence with the E1b promoter designated G5-E1b.

Fig. 4 shows SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of [^{35}S]-methionine labeled REA (top panel) incubated with GST-L540Q ER or GST alone bound to Sepharose beads in the presence of T (trans-hydroxytamoxifen), E (estradiol) or - (no ligand control). Protein eluted from Sepharose was analyzed by SDS-PAGE. In the lower panel, either [^{35}S]-methionine labeled L540Q-ER or wild-type (WT) ER was incubated with GST-REA bound to Sepharose beads. Bound protein was eluted from the beads and analyzed by SDS-PAGE. Molecular marker positions are shown to the right of each panel.

Fig. 5 is a set of bar graphs showing relative expression levels of an estrogen-responsive CAT reporter gene, controlled by ER, in the presence of SRC-1 or SRC-1 together with REA. The relative activity of the CAT reporter expression is given on the vertical axis. Levels of SRC-1 transfection vector are shown on the horizontal axis. REA was present at the indicated amounts (of transfection vector expressing REA) for the data shown in the middle and right hand panels.

Fig. 6 shows autoradiograph of SDS-PAGE gels of [^{35}S]- labeled REA (37 kDa) and SRC-1 (160 kDa) bound to GST-immobilized ER. Bars in the lower part of the figure represent quantitation of the respective labels based on autoradiogram density relative to 20% input controls. The components of each binding experiment are represented at the top of each column, with molecular weights and relative quantity of labeled component (35S-SRC-1 or 35S-REA) on the upper and lower parts of the vertical axis, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The estrogen receptor (ER) is a hormone-activated transcription factor that mediates the biological effects of estrogens in breast cancers and in a variety of other target tissues. In these tissues, the ER stimulates the expression of specific estrogen-regulated genes [for reviews, see Katzenellenbogen, B.S., (1996) *Biol. Reprod.* **54**:287; Santen, R. et al. (1990) *Endocr. Rev.* **11**:221; Sarrel, P.M. et al. (1994) *Sci. Am. Sci. Med.* **1**:44; Dickson, R.B. et al. (1987) *Endocr. Rev.* **8**:29; Katzenellenbogen, B.S. et al. (1997) *Breast Cancer Res. Treat.* **44**:23]. Because of the role of estrogens in promoting the growth and progression of breast cancers

[Dickson, R.B. et al (1987) *supra*; Katzenellenbogen, B.S. et al. (1997) *supra*], there is currently great interest in exploring ways to functionally inactivate the ER so as to suppress ER-mediated gene expression and cell proliferation. These approaches have involved the use of antiestrogens such as tamoxifen as well as dominant negative estrogen receptors. Antiestrogens compete with estrogens for binding to the ER but fail to activate the ER, and they are widely used in breast cancer treatment [Dickson, R.B. et al (1987) *supra*; Katzenellenbogen, B.S. et al. (1997) *supra*; Jordan, V.C. et al. (1990) *Endocr. Rev.* **11**:578]. Dominant negative ERs are slightly altered forms of the ER protein which lack transcriptional activity and which suppress the activity of the wild type ER when they are coexpressed in the same cells [Ince, B.A. et al. (1993) *J. Biol. Chem.* **268**:14026; Ince, B.A. et al. (1995) *J. Biol. Chem.* **270**:31163].

We previously identified dominant negative ERs altered in the C-terminal, helix 12 region of the ligand binding domain of the receptor through structure-function mutagenesis analysis [Ince, B.A. et al. (1993) *supra*]. Further studies on the mechanism of action of these dominant negative ERs indicated that their inhibitory effectiveness depended on their heterodimerization with the wild type ER and competition for DNA binding to estrogen response elements. However, their repressive effectiveness was greater than could be accounted for by only these two mechanisms, implying that their activity likely involved transcriptional silencing [Ince, B.A. et al. (1995) *supra*].

We hypothesized that the dominant negative ER, or an antiestrogen liganded-ER, might recruit into the dominant negative ER complex a repressive protein. To identify potential repressors of the estrogen receptor, we used the C-terminal E-F domains of the dominant negative mutant ER L540Q as "bait" in 2-hybrid screening in yeast [Fields, S. et al. (1989) *Nature* **340**:245] to identify clones from a MCF7 human breast cancer cell line cDNA library that express protein(s) that interact with the mutated AF-2 domain of the dominant negative ER. A plasmid encoding a chimeric protein consisting of the GAL4 DNA binding domain and the ER activation domain 2 with a mutation at amino acid 540, GAL(DBD)-ER (EF_{L540Q}), was contransformed with a cDNA library from MCF7 breast cancer cells, expressed as a translational fusion with the GAL4 transactivating domain (GAL(AD)-cDNA), into the YRG2

yeast strain. This yeast strain expresses two reporter genes, a histidine auxotrophic marker (*HIS3*) and a *LacZ* reporter gene, under the control of the GAL4 upstream activating sequence (UAS_{GAL4}). ER-interacting clones were then isolated from His synthetase gene positive clones (growth in media lacking histidine, *HIS3*$^+$) or clones that show increased transcription from the *LacZ* reporter gene (*LacZ*$^+$) (10). The transformed YRG2 yeast cells were selected for growth on plates in the presence of estradiol or the antiestrogen *trans*-hydroxytamoxifen (TOT) (10).

In this way, we isolated a clone, which we named REA for "Repressor of Estrogen receptor Activity." The interaction of REA with the ER fusion protein was confirmed by backcrossing. The interaction was found to be ligand-dependent, in that the activation of transcription from *LacZ* or *HIS3* reporter genes was observed only in the presence of antiestrogen or estradiol, and it occurred preferentially in the presence of antiestrogen.

The REA insert in GAL(AD) was sequenced, and this sequence (~700 bp from the 3' and coding regions) was compared to the gene databank using the BLAST search program. It was found to have 99% homology with a larger EST clone, which we obtained from the IMAGE Consortium. Sequence analysis of this 1500 bp cDNA clone indicated an open reading frame of 897 bp SEQ ID NO:1 (299 amino acids SEQ ID NO:2, Table 1), which contains a potential nuclear localization sequence and nuclear receptor interaction box (LXXLL motif). The sequence described herein has been deposited in the GenBank database, Accession No. AF150962. The clone shares nearly complete identity (99%) with the gene for a murine protein named B-cell receptor associated protein (BAP-37), originally isolated through its physical association with the B lymphocyte IgM antigen receptor [Terashima, M. et al. (1994) *EMBO J.* **13**:3782; Ansari-Lari, M.A. et al. (1997) *Genome Res.* 7:268]. The human ortholog of BAP-37 has recently been localized on chromosome 12 [Terashima, M. (1994) *supra*; Ansari-Lari, M.A. et al. (1997) *supra*]. REA also shows considerable relatedness to prohibitin (49% amino acid identity), a ubiquitously expressed regulatory protein [Kim, K.-M et al. (1994) *EMBO J.* **13**:3793; McClung, J.K. et al. (1995) *Exper. Gerontol.* **30**:99].

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    ATG GCC CAG AAC TTG AAG GAC TTG GCG GGA CGG CTG CCC GCC GGG
1   M   A   Q   N   L   K   D   L   A   G   R   L   P   A   G
    CCC CGG GGC ATG GGC ACG GCC CTG AAG CTG TTG CTG GGG GCC GGC
16  P   R   G   M   G   T   A   L   K   L   L   L   G   A   G
                                    NR
    GCC GTG GCC TAC GGT GTG CGC GAA TCT GTG TTC ACC GTG GAA GGC
31  A   V   A   Y   G   V   R   E   S   V   F   T   V   E   G
    GGG CAC AGA GCC ATC TTC TTC AAT CGG ATC GGT GGA GTG CAG CAG
46  G   H   R   A   I   F   F   N   R   I   G   G   V   Q   Q
    GAC ACT ATC CTG GCC GAG GGC CTT CAC TTC AGG ATC CCT TGG TTC
61  D   T   I   L   A   E   G   L   H   F   R   I   P   W   F
    CAG TAC CCC ATT ATC TAT GAC ATT CGG GCC AGA CCT CGA AAA ATC
76  Q   Y   P   I   I   Y   D   I   R   A   R   P   R   K   I
                                            NLS     PKA
    TCC TCC CCT ACA GGC TCC AAA GAC CTA CAG ATG GTG AAT ATC TCC
91  S   S   P   T   G   S   K   D   L   Q   M   V   N   I   S
    CTG CGA GTG TTG TCT CGA CCC AAT GCT CAG GAG CTT CCT AGC ATG
106 L   R   V   L   S   R   P   N   A   Q   E   L   P   S   M
    PKC
    TAC CAG CGC CTA GGG CTG GAC TAC GAG GAA CGA GTG TTG CCG TCC
121 Y   Q   R   L   G   L   D   Y   E   E   R   V   L   P   S
    ATT GTT AAT GAG GTG CTC AAG AGT GTG GTG GCC AAG TTC AAT GCC
136 I   V   N   E   V   L   K   S   V   V   A   K   F   N   A
    TCA CAG CTG ATC ACC CAG CGG GCC CAG GTA TCC CTG TTG ATC CGC
151 S   Q   L   I   T   Q   R   A   Q   V   S   L   L   I   R
                    PKC
    CGG GAG CTG ACA GAG AGG GCC AAG GAC TTC AGC CTC ATC CTG GAT
166 R   E   L   T   E   R   A   K   D   F   S   L   I   L   D
                    PKC
    GAT GTG GCC ATC ACA GAG CTG AGC TTT AGC CGA GAG TAC ACA GCT
181 D   V   A   I   T   E   L   S   F   S   R   E   Y   T   A
    GCT GTA GAA GCC AAA CAA GTG GCC CAG CAG GAG GCC CAG CGG GCC
196 A   V   E   A   K   Q   V   A   Q   Q   E   A   Q   R   A
    CAA TTC TTG GTA GAA AAA GCA AAG CAG GAA CAG CGG CAG AAA ATT
211 Q   F   L   V   E   K   A   K   Q   E   Q   R   Q   K   I
    GTG CAG GCC GAG GGT GAG GCC GAG GCT GCC AAG ATG CTT GGA GAA
226 V   Q   A   E   G   E   A   E   A   A   K   M   L   G   E
    GCA CTG AGC AAG AAC CCT GGC TAC ATC AAA CTT CGC AAG ATT CGA
241 A   L   S   K   N   P   G   Y   I   K   L   R   K   I   R
    GCA GCC CAG AAT ATC TCC AAG ACG ATC GCC ACA TCA CAG AAT CGT
256 A   A   Q   N   I   S   K   T   I   A   T   S   Q   N   R
    ATC TAT CTC ACA GCT GAC AAC CTT GTG CTG AAC CTA CAG GAT GAA
271 I   Y   L   T   A   D   N   L   V   L   N   L   Q   D   E
    AGT TTC ACC AGG GGA AGT GAC AGC CTC ATC AAG GGT AAG AAA TGA
286 S   F   T   R   G   S   D   S   L   I   K   G   K   K   *
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Table I

Based on the structural and functional characterizations of human REA described herein, a useful array of products and processes have become available to the art, either directly or through the predictable results of the application of ordinary skill in the art, all of which products and processes fall within the scope of the present invention. Given that mammals respond in similar ways to estrogen, the existence of other repressors of estrogen receptor activity in humans and in other mammalian species can be demonstrated using the techniques described herein. Furthermore, such repressors (termed REA generically) can be isolated and purified and their amino acid sequences, as well as the nucleotide sequences encoding them, can be determined by known techniques. While sequence variants can be isolated, such variants share regions of conserved sequence, particularly for those parts of the protein that are essential to, or contribute to its function as a repressor of estrogen receptor activity. The location and structure of the conserved regions can serve as a criterion for identifying an REA. The segments of human REA amino acids 19-49 and 150-174 are required for maximal repressor of ER activity. A protein having REA activity will be conserved within the segments corresponding to amino acids 19-49 and 150-174 of human REA. Alterations of amino acid sequence within these segments can be made, leading to variants of human REA having either reduced affinity, or increased affinity for ER, as desired. All such variants and alternatives having REA activity and having at least 60% amino acid identity within the segments corresponding to amino acids 19-49 and 150-174 of human REA lie within the scope of the invention. Variants having at least 80% amino acid identity are preferred, while variants having at least 90% are more preferred.

Antibodies, either polyclonal or monoclonal, or single chain antibodies, can be generated by known methods as for example those described by Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratories. Antibodies can be generated by using solubilized REA as an antigen, or by using a peptide having the amino acid sequence of a specific functional region of REA such as amino acids 19-49 and 150-174, or by immunizing with an REA fusion protein such as REA-oligo His, or GST-REA. Antibody preparations can be purified and cleared of non-specific binding antibodies by methods known in the art. Antibodies to denatured or unfolded REA can specifically bind to native REA. By binding specifically to an REA or a conserved epitope of an REA, such

antibodies can inhibit REA activity. Such antibodies can also be used to assay levels of REA in tissues, including tumor tissues. Tumor tissues identified as expressing a significant REA level are more strongly inhibited by antiestrogens, or by a dose that reduces the incidence of side effects. An antibody-based assay for REA activity can be useful for diagnosis of a pathological condition associated with a defect in REA function or other REA insufficiency. A variety of antibody-based assays are known in the art, many of which can be adapted for measurement of REA, as will be understood by those skilled in the art. The choice of assay will depend upon the level of sensitivity and convenience desired. All such assays are based upon measurement of antibody-bound REA or of the amount of an antibody-REA complex. Such assays include, but are not limited to, an ELISA assay or Western blot assay or IHC (immunohistochemical) assay. Accordingly, it will be recognized by those skilled in the art that an antibody with binding specificity to REA can have attached thereto any of a variety of label moieties including, but not limited to, a radioisotope, a fluorophore, a chromophore, a luminescent molecule, an enzyme or other detectable entity, according to the type of immunoassay desired. The enzyme-linked immunosorbant assay (ELISA) and Western blot and immunohistochemical assay are all standard assays and are currently used, for example, in measuring the level of estrogen receptor in breast tumor samples. The ELISA and Western blot assays use extracts from tissue homogenates whereas the immunohistochemical assay are performed on tissue sections. All of these are standard, commonly used assays, and would utilize antibodies which have been made to REA. The methods listed below, all of which utilize the commercially available estrogen receptor assay kit from Abbott Laboratories for estrogen receptor determination in breast tumors, can be readily adapted for measurement of REA.

1. Dittadi, R., Meo, S., Amoroso, B. and Gion, M., "Detection of different estrogen receptor forms in breast cancer cytosol by enzyme immunoassay." (1997) *Cancer Res.*, 57:1066-1072.

2. Karayiannakis, A.J., Bastounis, E.A., Chatzigianni, E.B., Makro, G.G., Alexiou, D., and Karamanakos, P. Immunohistochemical detection of oestrogen

receptors in ductal carcinoma in situ of the breast." (1996) *Eur. J. Surg. Oncol.* **22**:578-582.

3. Nedergaard, L., Christensen, L., Rasmussen, B.B., and Jacobsen, G.K., "Comparison of two monoclonal antibodies for the detection of estrogen receptors in primary breast carcinomas." (1996) *Pathol. Res. Pract.* **192**:983-988.

4. Goulding, H., Pinder, S.l, Cannon, P., Pearson, D., Nicholson, R., Snead, D., Bell, J., Elston, C.W., Robertson, J.F., Blamey, R.W., "A new immunohistochemical antibody for the assessment of estrogen receptor status on routine formalin-fixed tissue samples." (1995) *Hum. Pathol.*, **26**:291-294.

5. Nedergaard, L., Haeslev, T.,and Jacobsen, G.K., Immunohistochemical study of estrogen receptors in primary breast carcinomas and their lymph node metastases including comparison of two monoclonal antibodies." (1995) *Apmis.* **103**:20-24.

6. Saccani Jotti, G., Johnston, S.R., Salter, J., Detre, S. and Dowsett, M., "Comparison of new immunohistochemical assay for oestrogen receptor in paraffin wax embedded breast carcinoma tissue with quantitative enzyme immunoassay." (1994) *J. Clin. Pathol.* **47**:900-905.

7. Looi, L.M., Yap, S.F. and Cheah, P.L., correlation between oestrogen receptor protein expression in infiltrating ductal carcinoma of the breast by immunohistochemistry and cytosol measurements." (1997) *Ann. Acad. Med. Singapore* **26**:750-753.

Levels of REA expressed in tissue can also be measured by measuring the level of REA-mRNA in a sample of cells or tissue. For example, in the known technique of Northern blotting, labeled REA-cDNA is used as a hybridization probe against an mRNA preparation separated according to size on Gel electrophoresis.

The functional characteristics of REA can be used for a screening assay to identify compounds having estrogen or antiestrogen activity. REA binding to ER is ligand-dependant; either an estrogen or antiestrogen must be present for REA binding to ER.

An antiestrogen screening assay involves monitoring protein-protein interaction in which the ability of compounds to promote interaction of REA with estrogen receptor, but not to promote the interaction of SRC-1 (the steroid receptor coactivator-1) with estrogen receptor, is measured. To monitor REA interaction with estrogen receptor, an assay can be used that monitors the interaction of these two proteins as promoted by an antiestrogen, such as a GST-pulldown assay as described herein. Example 4 and Fig. 4. GST (glutathione-S-transferase) pulldown utilizes GST-estrogen receptor ligand binding domain fusion protein and an appropriately radiolabeled REA (such as 35S) or radiolabeled SRC-1, with interaction monitored in the presence and absence of compound being screened for antiestrogen activity. Antiestrogens recruit REA to estrogen receptor, but not SRC-1 to estrogen receptor. (See Example 6, Fig. 6.)

Related assays of protein interaction include scintillation proximity assays, surface plasmon resonance, and fluorescence resonance energy transfer (FRET). In the Scintillation proximity assay, estrogen receptor is immobilized on the surface of a scintillant by an appropriate technology (such as through an antibody against GST) and the ligand-induced proximity of radiolabeled REA to the estrogen receptor is assayed by an increased count rate. In the Surface Plasmon Resonance Assay, estrogen receptor is tethered to the sensor surface by an appropriate technology, and the ligand-induced interaction with REA is determined by a change in the signal. In the FRET assay, the ligand-induced interaction of the estrogen receptor with REA is followed by an increase in the FRET between the estrogen receptor labeled with one fluorophore through an appropriate technology (such as by interaction of GST-estrogen receptor with a flourescent labeled anti-GST antibody) and REA labeled with another fluorophore through an appropriate technology (such as by interaction of histidine-tagged-REA with a flourescent labeled anti-histidine-tagged antibody). The FRET reagents for epitope tagging REA or SRC-1 are all available commercially from Packard Bioscience Company. In each of these assays, the role (i.e., which partner is tethered) or labeling strategy

(i.e., which partner is radiolabeled or fluorescent labeled) of the estrogen receptor and REA (or SRC-1) can be reversed.

REA-estrogen receptor interaction by surface plasmon resonance can be done using a Biacore instrument [from Biacore AB Company, Stockholm, Sweden, as described in Cheskis, B., et al. (1996) *Biochemistry* 35:3309-3318, 1996 and Cheskis, B. et al. (1994) *Mol. Cell. Biol.* 14:3329-3338, for studying vitamin D receptor and RXR receptor interaction with DNA.] The FRET methodology to be employed has been utilized recently in studying the ligand-dependent interactions of other nuclear receptors with nuclear receptor coactivators [Zhou, G., et al. (1998) *Mol. Endocrinol.* 12:1594-1604. Each of these assays is based on our observations that antiestrogens only promote the interaction of REA (but not SRC-1) with estrogen receptor, while estrogens promote interaction of both SRC-1 and REA with estrogen receptor. Therefore, in screening for the ability of new compounds to act as an antiestrogen, estrogens are screened out, since estrogens promote the interaction of estrogen receptor with SRC-1.

The expression of REA in a desired host cell is an important aspect of the invention. Synthesis of REA for the purpose of isolation and purification depends upon having a suitable combination of a promoter operatively linked to a nucleotide sequence encoding REA. In addition, the present invention provides an opportunity to inhibit the growth of certain tumor cells by transforming the cells to express REA at elevated levels. Certain cancers, e.g., breast cancer, are stimulated by estrogens and inhibited by antiestrogens. By elevating the REA levels of such cells, the inhibitory effects of antiestrogens can be significantly amplified. Vectors for transformation and expression according to the present invention are an important component of a therapeutic transformation system. Selection of a promoter is governed by principles known in the art taking advantage of known functional attributes of a given promoter. Such attributes include, but are not limited to, whether the promoter drives constitutive expression, cell or species compatibility, whether the promoter is regulatable and, if so, by what means. Promoters are currently available that offer a wide range of such attributes, and more such promoters are being continually disclosed. Additional components regulating expression including upstream activating sequences, elements responsive to certain

environmental conditions, 3' or 5' untranslated regions, transcription termination signals and the like can also be incorporated into a vector for expressing REA, as known and understood in the art, given the desired goals for the amount of expression, type of regulation, and host cell specificity that one skilled in the art may wish to achieve.

The term "REA" is used to stand for repressor of estrogen receptor activity, a protein of about 37 kDa having unique functional properties, which acts to potentiate or amplify the repressive effects of antiestrogen. Human REA is exemplified. An REA (including an REA of another mammalian species, or a modified human REA) has functional properties as described herein and physical properties similar to human REA, especially in the region corresponding to amino acids 19-49 and 150-174 of human REA.

Activity as a repressor of estrogen receptor means the property of potentiating or amplifying the repression by an antiestrogen of an estrogen-regulated gene. REA activity can be measured by any of the methods exemplified herein, or by any other method for comparing the expression level of an estrogen-regulated gene in the presence and absence of an antiestrogen and in the presence and absence of an REA. In the absence of REA, a given concentration of an antiestrogen reduces the expression level of an estrogen-regulated gene by a measured amount. Under the same conditions except for the presence of REA, either the same concentration of antiestrogen produces a lower level of expression, or the reduced expression level is obtained at a lower concentration of antiestrogen.

A segment "corresponding to" a segment of human REA is a part of a non-human or modified human REA having the same general location on the molecule, and being associated with the same general functional attributes. For example, amino acids 19-49 and 150-174 of human REA have corresponding segments in other mammalian REA in approximately the same locations, taking account of the possibility of variation in over-all length as well as deletions or insertions within non-essential regions. Other structural features such as sequence motifs, disulfide bonds, sites or postranslational modification, sites of protease susceptibility and the like can also provide useful reference points for identifying and delineating corresponding segments, as is well-known in the art.

"Amino acid sequence identity" refers to standard known methods for comparing amino acid sequences. Percent sequence identity is measured by making a best fit alignment of the sequences under comparison, then subtracting any deletions, insertions and mismatches to calculate the percent of identical amino acids of one sequence compared to another. Such comparisons are commonly aided by computer analysis, particularly for finding the best fit, using reference databases, e.g. BLAST.

The term "binding specificity" as applied to an antibody is used to denote the fact that antibody molecules themselves have generally similar structural features and are commonly characterized in the art either with respect to the antigen used to generate the antibody, or to the protein, with which the antibody reacts. The antibody function is to bind to the antigen or protein that includes the antigen in its structure, to form an antibody-protein complex. It is well-known how to purify antibody preparations to remove antibodies that cross-react with other proteins, so that antibodies that are selective for the desired protein are purified. The term "binding specificity" therefore includes not only binding affinity for the designated protein, but selectivity for that protein as well.

A "promoter" is that part of a gene which directs and stimulates transcription, Promoters typically lie upstream of a coding segment (proximal to the 5' end of the coding region). A large number of promoters are known and available to the art. Readily available constitutive promoters include commercial available promoters of cytomegaloviruses (CMV), SV-40, Rous sarcoma virus (RSV), and thymidine kinase. The herein described pS2 promoter is a useful constitutive promoter. Inducible promoters include promoters include metallothionein, tetracycline, and ecodysone-induced promoter, all available commercially. Commercial sources for promoters include Clontech, Palo Alto, CA (www.clontech.com), Stratagene (www.stratagene.com), Pharmacia (www.pnu.com) and Invitrogen (www.invitrogen.com), among others. The selection of a suitable promoter for expression of REA in a host cell can be carried out by anyone skilled in the art, taking into consideration the characteristics of the promoter and the desired level of expression as well as desired timing and circumstances of regulation. For a general review of promoters, see Chambon, P. et al. (1984) *Recent Prog. Horm. Res.* **40**:1-42. Inducible regulation of expression using the tetracycline-

responsive promoter is described by Gossen M. et al. (1992) *Science* 268:1766-1769. The pS2 promoter is described by Jeltsch, J.M. et al. (1987) *Nucl. Acids. Res.* 15:1401-1414, and other articles from the laboratory of P. Chambon.

A "transformation vector" is a genetic element that can be transferred into a desired host cell, and which carries genetic determinants for expressing a specified coding sequence within the host cell. Typically, the transformation vector is a plasmid or a modified viral DNA. Using any of the many known methods for *in vitro* or *in vivo* transformation, a transformation vector can be introduced into a host cell, within which the desired gene is expressed under the desired conditions. The choice of transformation vector is governed by principles known in the art and depends upon such factors as the type of cell to be transformed, the desired transformation efficiency, and the like.

In the ensuing examples, the following materials and methods were employed.

Chemicals and Materials. The antiestrogens ICI182,780 (ICI) and *trans*-hydroxytamoxifen (TOT) were kindly provided by Alan Wakeling and Zeneka (Wilmington, De). Custom oligonucleotides were purchased from GIBCO.

Plasmids. pBD-GAL4-EF was constructed by subcloning the blunted *Eag*I/*Bam*HI insert from pCMV5-ER (wild type or LF40Q mutant) into the *Eco*RI-digested and blunted pBD-GAL4 (Stragene). pCMV-REA was constructed by releasing REA cDNA from the pT7T3D-PAC vector by *Xho*I/*Not*I digest. The insert then was blunted and subcloned into *Sal*I/*Sma*I-digested and blunted CMV5. The pCMV5 expression vector for the wild-type human ER (WT-ERα), the dominant negative ER L540Q [Fields, S. et al. (1989) *supra*; Terashima M. et al. (1994) *supra*], human ERβ (530 residues), and human progesterone receptor, pCMV5-PR_B, have been described. The expression vectors pRSV-RARα and pBK-CMV-SRC-1, pCMV-androgen receptor, and human thyroid receptor β1 were obtained from Ron Evans (Salk Institute, La Jolla, CA), Bert O'Malley (Baylor College of Medicine, Houston), Michael McPhaul (Southwestern Medical School, Dallas), and William Chin (Harvard Medical School, Boston).

The estrogen response element (ERE)-containing reporters $(ERE)_2$-TATA-Chloramphenicol acetyltransferase (CAT) and $(ERE)_2$-pS2-CAT, and the CMV-ERE-CAT promoter interference plasmids have been described previously [Terashima M. et al. (1994) *supra*; Wrenn, W.E. et al. (1993) *J. Biol. Chem.* 268:240889]. Mouse mammary tumor virus (MMTV-CAT) was obtained from Steven Nordeen (University of Colorado Medical Center, Denver). SV-DR5-CAT, the retinoic acid receptor (RAR)-responsive reporter, was obtained from Ronald Evans. G5-E1b0CAT reporter construct containing the Ga14 up-stream activating sequences (was obtained from Michael Green (University of Massachusetts, Worcester, MA). The plasmids pCH110 (Pharmacia) and pCMVβ (CLONTECH) were used as β-galactosidase internal controls for transfection efficiency, and all CAT activity measurements were corrected for β-galactosidase activity [Terashima M. et al. (1994) *supra*; McInerney, E.M. et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:10069].

To make truncated REA constructs, deletions of the REA ORF were generated by PCR using the full-length REA plasmid, pCMV-REA, as template. Reactions with constructed forward and reverse PCR primers were performed by using VENT DNA polymerase from new England Biolabs. Each forward primer contained an ATG and identical Kozac sequence, and each reverse primer contained a stop codon. PCR fragments were purified, digested with *Eco*RI and *Xba*I, and cloned into the *ECO*RI/*Xba*I sites of pCMV5.

cDNA Library Construction. cDNA was prepared from MCF-7 cells and ligated into the HybriZAP vector (Stratagene) to generate a primary λ library. This library was amplified and converted by *in vivo* excision to a pAD-GAL4 phagemid library. The average insert size in the phagemid library is 1.4 kb.

Yeast Two-Hybrid Screening. The yeast strain YRG2 (Stratagene) containing pBD-GAL-EF (wild type or L540Q, amino acids 313-595) was transformed with the human MCF-7cDNA library in pAD-GAL4 and plated on medium lacking histidine and supplemented with 10^{-5} M TOT. *HIS3*$^+$ colonies were measured for β-galactosidase activity by using the filter-lift assay [Montano, M.M. et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:2581]. *HIS3*$^+$ colonies exhibiting high β-galactosidase activity (*LacZ*$^+$ colonies) were characterized further. To

recover library plasmids, total DNA from *HIS3*$^+$, *LacZ*$^+$ colonies was isolated and used to transform *Escherichia coli* (XLI-Blu MRF' strain from Stratagene). To ensure that the correct cDNAs were identified, as well as to establish ligand-dependent interaction of promising clones with the ER, library plasmids isolated were transformed into YRG2 containing pBD-GAL-EF and plated into medium lacking histidine and supplemented with control vehicle, estradiol (10^{-5} M), or TOT (10^{-5} M). β-Galactosidase activity was determined from *HIS3*$^+$ colonies by using both the filter-lift assay or liquid assay [Montano, M.M. et al. (1997) *supra.*].

Cell Culture and Transfection. MCF-7 human breast cancer cells, Chinese hamster ovary (CHO) cells, and MDA-MB-231 human breast cancer cells were maintained in cell culture and transfected by the $CaPO_4$ coprecipitation method exactly as described previously [Terashima, M. et al. (1994) *supra*]. CHO cells were plated at 1.8×10^5 per 60-mm plate and transfected 48 h later with 2 μg pCH110, 5 ng pCMV5-ER expression vector, and carrier DNA to 8 μg total DNA per plate. MDA-MB-231 cells in 100 mm dishes were transfected with 6 μg of the ERE-containing reporter construct [$(ERE)_2$-pS2-CAT], 100 ng CMV5-ER expression vector, 1 μg pCMVβ, β-Galactosidase activity, which was measured to normalize for transfection efficiency, and CAT activity were assayed as described [Terashima, M. et al. (1994) *supra*].

In Vitro Translation. *In vitro* translation of REA, ER, or other receptors was performed [McInerney, E.M. et al. (1996) *supra*] by using the Promega TNT kit.

In Vitro Interaction Assays. Five hundred micrograms of *E. coli* bacterial crude extract containing glutathione *S*-transferase (GST)-ER hormone-binding domain (amino acids 282-595) or GST-REA fusion proteins was incubated at 4°C with 25 μl of glutathione-Sepharose beads (50% slurry; Pharmacia) for 2.5 h as described (18) with minor modifications. After two washes with 1 ml NET (20 mM Tris, pH 7.9/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40/0.5% milk) and two washes with 1 ml binding buffer (20 mM DTT/6 mM $MgCl_2$/1 mM EDTA), the beads were incubated with 5 μl of *in vitro* translated product for 2.5 h at 4°C. The beads then were washed three times with 1 ml NET and two times with 1 ml binding buffer. After washing, bound protein was eluted with 10 mM reduced glutathione in 50 mM

Tris HCl, pH 8.0, and boiled in SDS sample buffer. One-fourth of each protein sample was analyzed by SDS/PAGE. The gel was dried, and [^{35}S]methionine-radiolabeled protein was detected by autoradiography.

Human REA was isolated and purified. An REA-oligo His fusion protein was expressed in host cells, then purified from cell lysate by passage over a nickel column, (nicke-charged nitrotriacetic acid-agarose resin; Ni-NTA-agarose; Quiagen, Inc. Santa Clarita, CA) according to standard protocols [as in K.E. Carlson, et al. (1997) *Biochemistry* **36**:14897-14905].

Example 1:

Regions of human REA required for repression of ER activity were mapped by measuring the ability of various amino- or carboxyl-terminal truncated forms of REA to repress the ability of ER to affect expression of a reporter gene (CAT) transcribed from an estrogen-responsive promoter MDA-MB-231 human breast cancer cells were co-transfected with 100 ng pCMV-ERα, and 500 ng of pCMV-REA (full-length or truncated) as well as the $(ERE)_2$-pS2-CAT reporter and an internal control β-galactosidase plasmid as an internal standard. Cells were treated with 10^{-8}M E_2 for 24 h. The level of repression of ER activity by full-length REA was set as 100%. The effectiveness of the truncated forms of REA for repressing ER activity is shown in Fig. 1 as a percentage of full-length REA. Values are the means ±SD of three to eight determinations.

As shown in Fig. 1, deletion of the first 18 or the last 125 amino acids of REA had almost no effect of repression of ER activity. However, further N-terminal deletion to amino acid 49 or C-terminal deletion to amino acid 150 resulted in complete inability of REA to repress ER activity. (Although REA 1-226 appeared to be only partially active, it was able to suppress ER activity to the same level as full-length REA when higher amounts of REA expression plasmid were utilized.) Thus, repressive activity of REA requires two regions of REA encompassing amino acids 19-49 and 150-174.

Example 2.

The effect of REA on the ability of the dominant negative ER to suppress wild type ER activity in mammalian cells was examined. REA was expressed in CHO cells from the mammalian expression vector pCMV5 along with the hormone-responsive reporter gene construct ERE_2-TATA-CAT and an expression vector for the indicated receptor or transcription factor. In obtaining the data shown in Figs. 2A-C, CHO cells were transfected with 5ng of ER expression vector (described, *supra*) and the CAT reporter construct, in the presence or absence of 10 ng REA expression vector. For the data of Fig. 2A, cells were cotransfected with increasing amounts, as indicated, of the expression vector for the dominant negative L540QER (described, *supra*) and were treated with $10^{-8}ME_2$ for 24 h. In obtaining the data of Figs. 2B and 2C, cells were treated with $10^{-8}ME_2$ along with increasing concentrations of the indicated antiestrogen, either TOT (Fig. 2B) or ICI 182,780 (Fig. 2C) for 24 h. For the data of Fig. 4D, CHO cells were transfected with 5 ng of the expression vector for human progesterone receptor (PR) (described, *supra*), the PR responsive reporter construct MMTV-CAT (described, *supra*), in the presence or absence of 10 ng REA expression vector, and were treated with 10^{-8}M progestin R5020, plus increasing amounts, as indicated, of the antiprogestin RU486 for 24 h. As in Example 1, all cells were transfected with a β-galactosidase internal control reporter to correct for variations in transfection efficiency. Cell extract CAT activity values, normalized for β-galactosidase activity, are the means ±SD from three separate experiments.

As shown in Fig. 2A, REA markedly potentiated the inhibitory effectiveness of the dominant negative L540Q ER. For these experiments, wild type ER was expressed in CHO cells with increasing amounts of the dominant negative ER in the presence or absence of REA. A very low concentration of REA (10 ng expression plasmid) reduced by 4-fold the amount of dominant negative ER required to suppress 50% of wild type ER activity. As shown in Fig. 2B and 2C, REA also markedly potentiated the inhibitory activity of antiestrogens. Intriguingly, REA at a very low level (10 ng expression plasmid) resulted in a 50-fold increase or leftward shift in the inhibitory potency of the antiestrogen trans-hydroxytamoxifen (TOT) and the antiestrogen ICI 182,780 (ICI). Thus, 50-fold less antiestrogen is needed for equal inhibitory effectiveness in the presence of low amounts of REA.

The small amount of REA expression vector that elicited this marked enhancement of L540Q ER dominant negative effectiveness and suppression by antiestrogens caused little if any decrease in activity of the wild type ER (see zero point values in Panels 2A, B, or C), indicating that the potentiation of inhibitory activity of the dominant negative ER or antiestrogen-liganded ER cannot be attributed to a decrease in the activity of the wild type ER alone. The data in Fig. 2D show that REA did not enhance the suppressive effects of antiprogestins on progesterone receptor (PR) transcriptional activity, indicating that the effects of REA are selective for the ER.

Example 3.

Transcriptional activity experiments shown in Fig. 3 further indicate that REA is an estrogen receptor-selective coregulator. For the data of Figs. 3A, 3B and 3D-F, CHO cells were transfected with 5 ng of the expression vector for various activators and 2 μg of the indicated reporter construct (all vectors and constructs previously described). Fig. 3A: activator = ERα, reporter = $(ERE)_2$-TATA-CAT; Fig. 3B: activator = ERβ, reporter = $(ERE)_2$-TATA-CAT; Fig. 3D: activator = PR, reporter = MMTV-CAT; Fig. 3E: activator = RAR, reporter = DR-5-CAT; Fig. 3F: activator = Gal4-VP-16, reporter = G5-E1-CAT. For the data of Fig. 3C, MDA-MB-231 breast cancel cells were transfected with 100 ng of the ERα expression vector and 6 μg of the $(ERE)_2$-pS2-CAT reporter. In all cases, the cells were transfected with increasing concentrations, as indicated, of an REA expression vector and with a β-galactosidase internal control reporter to correct for transfection efficiency. All cells were treated for 27 h with the indicated ligands at 10^{-8}M (ER; E_2; PR; R5020; RAR: all-trans-retinoic acid). Cell extracts were prepared and assayed for CAT activity, normalized for β-galactosidase activity. Values shown are the means $\pm$SD from three separate experiments. Numbers at the top leftmost bar of each panel show the fold induction in CAT activity by hormone in the absence of REA (receptor-transfected, plus vs. minus hormone).

When the estrogen responsive $(ERE)_2$-TATA-CAT reporter gene construct was transfected into estrogen receptor-negative CHO (Chinese Hamster Ovary) cells along with an expression vector for ERα or ERβ, addition of estradiol resulted in a 26-fold or 11-fold increase, respectively, in ER transcriptional activity. When REA and the ER were

coexpressed, transcriptional activation by the ER was suppressed in a dose-dependent fashion by REA (Fig. 3A and 3B). REA had no effect on reporter activity in the absence of liganded ER. REA-mediated repression of ER transcriptional activity was also observed in other cells, such as MDA-MB-231 breast cancer cells and HEC-1 human endometrial cancer cells, and in the context of another promoter reporter construct $(ERE)_2$-pS2-CAT, indicating that REA does not appear to be a cell-or promoter-specific repressor.

In contrast to what was observed with ERα and ERβ, REA had little or no effect on the transcriptional activity of other hormone receptors, including progesterone receptor (PR, Fig. 3C), retinoic acid receptor (RAR, Fig. 3D), and androgen receptor or thyroid hormone receptors (data not shown), and it had no effect on the transcriptional activity of an unrelated transcriptional activator, Gal4-VP16 (Fig. 3E), even when amounts of REA much greater than those giving nearly full inhibition of ER activity were used in the same cells.

Example 4.

The interaction between REA and ER was verified *in vitro*. We used a protein-protein interaction assay in which the affinity matrix was a glutathione-S-transferase (GST) fusion protein wherein GST was fused to the AF2 domain of the human ER (amino acids 282-595), GST-ER. The data are shown in Fig. 4. For the data of the top panel, *in vitro*-translated [^{35}S]methionine-labeled REA was incubated with GST-L540Q-ER (lanes 1-3) or GST-alone bound to Sepharose beads (lane 4) in the presence of 0.1% ethanol control vehicle (-), 10^{-6}M *trans*-hydroxytamoxifen (T), or 10^{-6}M estradiol (E). For the data of the bottom panel, *in vitro*-translated [^{35}S]methionine-labeled L540Q ER (lanes 1-2) or wild type ER (lanes 3-4) were incubated with GST-REA (REA bound to GST-Sepharose beads) in the presence of control vehicle (-) or estradiol (E). Bound protein was eluted and analyzed by 12.5% SDS-polyacrylamide gel electrophoresis. The numbers at the left indicate molecular size markers in kilodaltons (kDa). Typically, 12-15% of input-radiolabeled wild-type ER was bound to GST-REA in the presence of ligand.

In vitro transcribed and translated [^{35}S]REA was retained by the GST-ER affinity column in the presence of TOT and estradiol (Fig. 4). REA was not retained on the GST

column without the ER AF2 domain (or with GST alone). Likewise, REA did not interact with the ligand binding domain of human PR-B (GST-PR) in the presence or absence of ligand (15). We compared the interaction of wild type ER and the L540Q mutant ER with REA in GST pull down assays wherein GST was fused to REA (GST-REA). As shown in Figure 4, higher levels of the dominant negative L540Q ER were retained on the GST-REA column when compared to the wild type ER, and the interaction with both receptors was greatly increased by hormone. These findings suggest that REA interacts preferentially with the dominant negative versus wild type ER.

Example 5.

We examined if REA inhibits ER action by interfering with the DNA binding function of the ER. The ability of the ER to bind to estrogen response element (ERE) DNA was tested *in vivo* using a promoter interference assay [Reese, J.C. et al. (1992) *Mol. Cell. Biol.* 12:4531]. CHO cells were transfected with 5 ng of the ER expression vector in the absence or presence of expression vector for REA (25, 50 or 100 ng) and 40 ng of the CMV-$(ERE)_2$-CAT promoter interference reporter construct containing either two wild type or mutated (mut) EREs. The cytomegalovirus promoter-estrogen response element-chloramphenicol acetyltransferase (CMV-ERE-CAT) promoter interference plasmids have been previously described [Reese, J.C. et al. (1992) *supra*]. After transfection, the cells were treated with 10^{-8} M estradiol for 24 h. Cell extracts were prepared and analyzed for CAT activity and β-galactosidase activity (17). The level of activity in cells which were transfected with the CMV-$(ERE)_2$-CAT reporter but no ER expression vector was set at 100%.

The described assay allowed us to examine the effect of REA on ER DNA-binding ability in intact cells using low levels of expression vector for REA. In this method, the binding of the ER to the ERE is assayed by the ability of the ER bound to the ERE to block transcription from a constitutively active cytomegalovirus promoter. A 57% repression of CMV promoter activity was observed in cells cotransfected with an expression vector for the wild type ER. Repression required a functional ERE and was not observed with a mutated form of the ERE (ERE mut) to which the ER does not bind. Cotransfection with REA did not significantly change the magnitude of ER-mediated repression of CMV promoter activity, and

REA alone did not affect the activity of the CMV promoter. It is thus clear that the repression of ER activity by REA is not due to a reduction in ER DNA-binding activity. Our data also implies that REA does not act by keeping the ER out of the nucleus.

We next determined if REA affects steps subsequent to DNA binding in the ER response pathway. One of these steps is the functional interaction of the ER with necessary adapter or coactivator proteins. CHO cells were transfected with 5 ng of expression vector for ER and 2 μg of $(ERE)_2$-TATA-CAT reporter construct. The cells were cotransfected with increasing concentrations of an expression vector for SRC-1 in the presence or absence of REA as indicated. The cells were also transfected with a β-galactosidase internal control reporter to correct for transfection efficiency. Cell extracts were then treated for 24 h with 10^{-8} M estradiol (E_2). Cell extracts were prepared and analyzed for CAT activity and β-galactosidase activity. Values are the means ± SD from three separate experiments.

As expected (Onate, S.A. (1995) *Science* **270**:1354; McInerney, E.M. et al. (1996) *supra*], the coactivator SRC-1 enhanced the estradiol-mediated transcriptional activity of ER up to 4-5-fold (Fig. 5, left hand panel). Coexpression of REA suppressed the enhancement of ER transcriptional activity by SRC-1, and it did so in a concentration-dependent manner (Fig. 5, middle and right panels), implying mutual competition between these two proteins and suggesting that REA and SRC-1 may compete for interaction with ER and for modulation of ER biological activity. Of note, much lower levels of the expression vector for REA (when compared to the expression vector for SRC-1) were needed to inhibit the SRC-1-mediated enhancement of ER transcriptional activity. It is known that SRC-1 binds to the activation function-2 (AF-2) region of nuclear receptors [Onate, S.A. et al. (1995) *supra*; Lazennec, G. et al. (1997) *Mol. Endocrinol.* **11**:1375], and our yeast 2-hybrid screening pulled out REA using the portion of the ER encompassing AF-2. This result indicates that the mutual competition between REA and SRC-1 represents competitive binding of both factors with the AF-2 region of the receptor, although further studies will provide greater resolution of the portions of REA and ER which interact. The observation that the L540Q dominant negative ER shows preferential interaction with REA is consistent with this hypothesis, as we find that this transcriptionally inactive mutant ER fails to interact with coactivators such as SRC-1.

Interestingly, REA enhances antiestrogen and dominant negative ER potency at concentrations that are lower than those at which it suppresses estradiol-ER activity. At higher concentrations of REA, where estradiol-stimulated activity of the wild type ER is suppressed, similar efficacy of REA is observed with ERα and ERβ. In Figure 5C, we present a model for REA potentiation of antiestrogen inhibitory effectiveness and for repression of ER activity. This model predicts that the cellular level of REA will be an important determinant of the effectiveness (potency) of antiestrogens in inhibiting estrogen activity: In cells with no or low levels of REA, estradiol will effectively activate ER-mediated transcription and antiestrogens will suppress estrogen action; at high cellular levels of REA, antiestrogen potency will be markedly enhanced, resulting in a much greater inhibitory effectiveness of low concentrations of antiestrogen. With high levels of REA, estradiol-occupied ERs will also have their stimulatory effectiveness suppressed, thereby further reducing estrogen activity in cells. REA thus sensitizes cells to the antagonist activity of antiestrogens over a range where it has minimal effect on desensitizing cells to estrogens, but at high levels, reduced response to estradiol would also be expected.

REA bears no structural resemblance to three known corepressors of the nuclear receptor subfamily of non-steroid hormone receptors, namely N-CoR, SMRT and SUN-CoR [Chen, J.D. et al. (1995) *Nature* **377**:454; Horlein, A.J. et al.,(1995) *ibid* **377**:397; Chen, J.D. et al. (1996) *Cell* **89**:373; Zamir, I. et al. (1997) *Proc. Natl. Acad. Sci. USA* **94**:14400], and unlike these corepressors, which interact with thyroid hormone or retinoic acid receptors, REA shows great selectivity for estrogen receptors. A major difference between REA and N-CoR/SMRT and SUN-CoR is that REA interacts preferentially with a liganded ER, whereas SMRT and N-CoR and SUN-CoR interact with an unliganded receptor and dissociate from the receptor upon ligand binding. Thus, repressor interaction with unliganded thyroid receptor or retinoic acid receptor correlates well with the repressive activity of thyroid receptor or retinoic acid receptor in the absence of their ligand. In contrast, the ER when unliganded is inactive and does not have repressive activity. Although SMRT and N-CoR have been shown to suppress the agonistic activity of the tamoxifen-occupied ER [Jackson, T.A. et al. (1997) *Mol. Endocrinol.* **11**:693], these corepressors do not enhance the inhibitory effectiveness of antiestrogens, nor do they alter the activity of estrogen-liganded ER. REA is involved in

potentiating the repression by the antiestrogen-liganded ER and dominant negative ERs and, at higher levels, in reduction of the transcriptional activity of estrogens.

Although REA is unrelated structurally to the three known corepressors of some nuclear receptors (SMRT, N-CoR, and SUN-CoR), it shows very strong homology with a murine B-cell receptor associated protein named BAP-37. Although first identified [Terashima, M. et al. (1994) *supra*; Ansari-Lari, M.A. et al. (1997) *supra*; Kim, K.-M. et al. (1994) *supra*] in B cells, BAP-37 was also shown to be present in all cells and tissues examined, clearly indicating that it functions in cells which do not contain the B cell receptor, although its activities have not been characterized [Terashima, M. et al. (1994) *supra*; Ansari-Lari, M.A. et al. (1997) *supra*]. Intriguingly, BAP-37 is related to prohibitin (49% amino acid identity), a highly conserved protein that is reported to play roles in diverse processes, including development, tumor suppression, and senescence [McClung, J.K. et al. (1995) *supra*; Jupe, E.R. et al. (1996) *Cell. Growth Diff.* **7**:871; Asamoto, M. et al. (1994) *Cancer Letters* **83**:201; Thompson, W.E. et al. (1997) *J. Reprod. Fert.* **109**:337; Liu, X.-T. et al. (1994) *Biochem. Biophys. Res. Commun.* **201**:409; Nuell, M.J. et al. (1991) *Mol. Cell. Biol.* **11**:1372; Sato, T. et al. (1993) *Genomics* **17**:762].

To the best of our knowledge, REA is the first estrogen receptor-selective coregulator to be identified. REA also represents the first example of a protein which enhances the potency of two inhibitors of ER action, antiestrogens and dominant negative ERs, implying that interaction with REA may represent an important point of convergence in the mechanism of action pathway through which these two factors function. The ER-selective activities of REA indicate that it plays an important role in determining the sensitivity of estrogen target cells, including breast cancer cells, to antiestrogens and estrogens.

Example 6.

The interactions of REA with ER, of SRC-1 with ER and the combination of REA and SRC-1, in the presence of an estrogen or an antiestrogen, were studied by analysis of GST-pulldown assays. Binding studies of GST-REA in the presence of various segments of ER, in the presence of TOT or E2 revealed that REA interacts with the carboxy-terminal portion of

the ER (domains E and F) in a hormone-dependent manner. Furthermore, the N-terminal A, B and C regions of domains of ER do not interact with REA. These findings made it possible to conduct other GST-pulldown assays using a GST labeled segment of ER, from amino acids 282-595 (GST-ER 282-595) GST-ER 282-595 was incubated with radiolabeled SRC-1 alone; with radiolabeled REA alone; and with a fixed amount of radiolabeled REA in the presence of increasing amounts of radiolabeled SRC-1. The results are shown in Fig. 6. In the rows bracketed "SRC-1" the data demonstrate that SRC-1 interacts with ER in the presence of estrogen (E_2) but not antiestrogen (TOT). The rows bracketed "REA" demonstrate that REA interacts with ER in the presence of E_2 or of TOT. In the rows bracketed as REA and SRC-1 (the latter at increasing concentrations from left to right), the data demonstrate that REA binding to ER is competitive with SRC-1 (in the presence of E_2).

Example 7.

The level of REA in MCF-7 breast cancer cells was measured in the presence and absence of either estrogen or antiestrogen. The cellular level of REA was neither increased nor decreased by estrogen or antiestrogen.

Levels of REA mRNA were analyzed in a large variety of samples of human tissues. The results demonstrated that REA is expressed in virtually all cell lines and tissues. Levels of mRNA were somewhat different in different tissues. REA was relatively high in liver, ovary, mammary gland, prostate, pituitary and small intestine, and lowest in the brain tissues examined.

The introduction of REA antisense RNA into breast cancer or ovarian cancer cells resulted in a 3- to 4-fold increase in response to estrogen, as measured by transcriptional activity of the ER in the presence of 10^{-9}M estradiol. Therefore, endogenous cellular REA normally dampens the tissue response to estrogen.

The invention has been described with respect to the data obtained for human REA and its functional attributes. It will be recognized by those of ordinary skill in the art that the principles and teachings herein can be more broadly applied toward an array of products and processes that further embody these principles and teachings. The invention therefore includes equivalent products, structures, methods, processes and assays as will be readily apparent to those skilled in the art and applied without undue experimentation, all as set forth in the claims.

WE CLAIM:

1. An isolated and purified protein having activity as a repressor of estrogen receptor (REA), having a molecular weight of about 37 kDA and having the amino acid sequence of human REA or an amino acid sequence at least 60% identical thereto within those segments of the protein corresponding to amino acids 19-49 and 150-174 of human REA.

2. The protein of claim 1 having at least 80% sequence identity within the segments corresponding to amino acids 19-49 and 150-174 of human REA.

3. The protein of claim 1 having at least 90% sequence identity within the segments corresponding to amino acids 19-49 and 150-174 of human REA.

4. The protein of claim 1 having the amino acid sequence of SEQ ID NO:2.

5. The protein of claim 1 having an added amino acid sequence combined therewith to form a fusion protein, the added sequence forming a specific binding site.

6. The fusion protein of claim 5 where in the added sequence is oligo-histidine.

7. The fusion protein of claim 5 wherein the added sequence is glutathione-S-transferase.

8. An antibody having binding specificity for a repressor of estrogen activity.

9. The antibody of claim 8 wherein the antibody is polyclonal.

10. The antibody of claim 9 having a label moiety attached thereto, the label moiety being selected from the group, a radioisotope, a fluorophore, a chromophore, a luminescence molecule or an enzyme.

11. The antibody of claim 8 wherein the antibody is monoclonal.

12. The antibody of claim 11 having a label moiety attached thereto, the label moiety being selected from the group, a radioisotope, a fluorophore, a chromophore, a luminescence molecule or an enzyme.

13. A method for measuring the amount of repressor of estrogen receptor activity (REA) in a cell or tissue sample, comprising the steps of

contacting a lysate or protein extract of said sample with an antibody having binding specificity for said REA, under conditions permitting formation of an antibody REA complex, the amount of said complex being proportionate to the amount of REA in said sample,

separating the antibody-REA complex from said sample,

measuring the amount of the antibody-REA complex,

comparing the amount of antibody-REA complex from said sample with an amount of antibody-REA complex from a control sample having a known or standardized amount of REA, whereby the amount of REA in said sample is measured.

14. The method of claim 13 wherein the method is an enzyme-linked immunoassay (ELISA).

15. The method of claim 13 wherein the method is a radioimmune assay (RIA).

16. The method of claim 13 wherein the method is an immunofluorescent assay.

17. A method of testing a compound for antiestrogen activity comprising the steps of

combining estrogen receptor (ER), or a segment thereof capable of binding a repressor of estrogen receptor activity (REA), with REA in the presence of the compound being tested under conditions permissive for binding to occur,

measuring the amount of labeled REA bound to ER then, if any binding is measured, combining ER or the segment thereof capable of binding REA, with SRC-1, in the presence of the compound being tested,

measuring the amount of SRC-1 bound to ER,

comparing the amount of REA bound to the amount of SRC-1 bound whereby the compound being tested has antiestrogen activity if the amount of binding of REA to ER exceeds the amount of SRC-1 binding to ER in the presence of the compound being tested.

18. The method of claim 17 wherein the binding of REA or of SRC-1 to ER is measured by surface plasmon resonance.

19. The method of claim 17 wherein REA or SRC-1 has a label moiety attached thereto, selected from the group: a radioisotope, a chromophore, a fluorophore or a luminescent compound.

20. The method of claim 19 wherein the label moiety is a radioisotope and ER is immobilized as a fusion protein with glutathione-S-transferase on a support matrix having glutathione covalently attached thereto.

21. The method of claim 19 where the label moiety is a radioisotope and binding is measured by scintillation proximity.

22. The method of claim 19 wherein the label moiety is a fluorophore and binding is measured by fluorescence resonance energy transfer.

23. A DNA comprising a promoter operatively linked with a nucleotide sequence encoding an REA, said promoter being capable of controlling transcription of the sequence encoding an REA in a desired host cell.

24. A transformation vector comprising the DNA of claim 23.

25. The vector of claim 24 wherein the nucleotide sequence is SEQ ID NO:1.

26. The vector of claim 23 wherein the nucleotide sequence additionally encodes an additional amino acid sequence combined therewith to encode a fusion protein, the added amino acid sequence forming a specific binding site.

27. The vector of claim 26 wherein the additional nucleotide sequence encodes oligo-histidine.

28. The vector of claim 26 wherein the additional nucleotide sequence encodes glutathione-S-transferase.

29. A method for potentiating the activity of an antiestrogen administered to a cell comprising the steps of

transforming the cell with a transformation vector comprising a segment encoding a repressor of estrogen receptor activity (REA) expressible in the cell, and

maintaining the cell under conditions that permit expression of REA in the cell, whereby administration of an antiestrogen to the cell increases the activity of the antiestrogen in the transformed cell compared to the activity of the antiestrogen in an untransformed cell.

30. The method of claim 29 wherein the segment encoding an REA is expressible under control of a constitutive promoter.

31. The method of claim 30 wherein the segment encoding an REA is expressible under control of an inducible promoter.

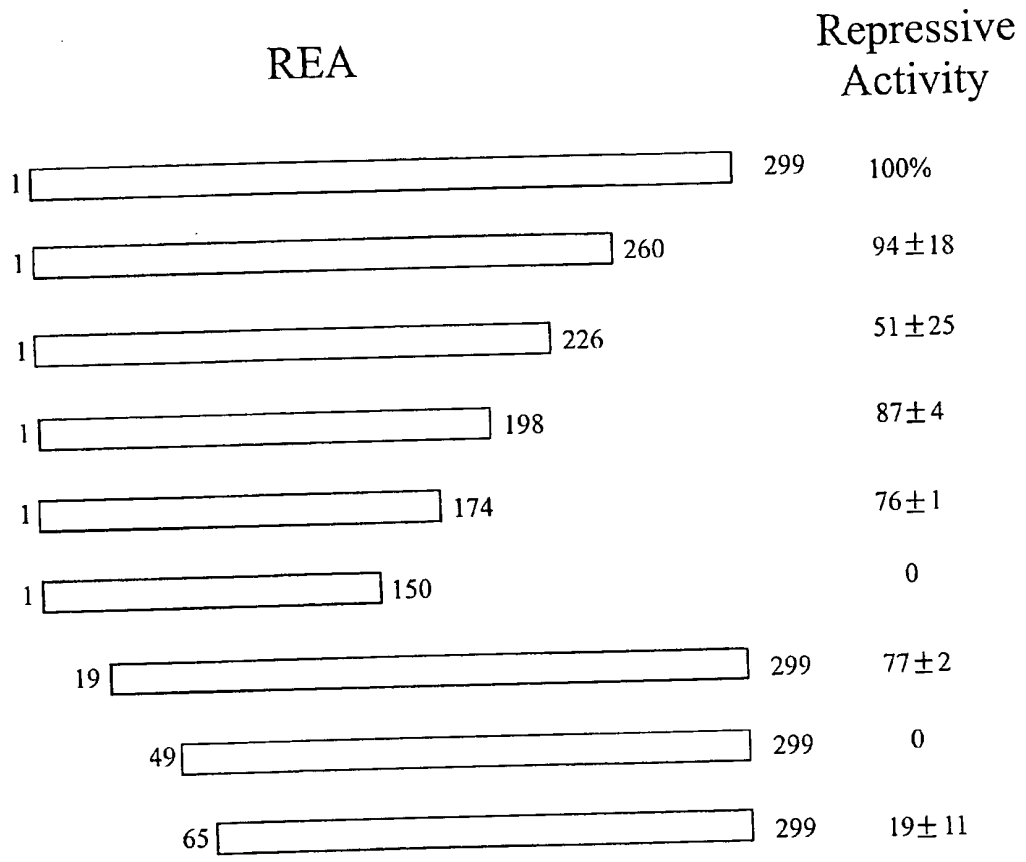
32. The method of claim 29 wherein the host cell is a breast cell.

33. The method of claim 29 wherein the host cell is an endometrial cell.

34. The method of claim 29 wherein the host cell is an ovarian cell.

35. The method of claim 32 wherein the host cell is a breast cancer cell.

36. The method of claim 29 wherein the segment encoding an REA has the nucleotide sequence of SEQ ID NO:1.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1
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1
226
51±25
1
198
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0
19
299
77±2
49
299
0
65
299
19±11

FIG. 1

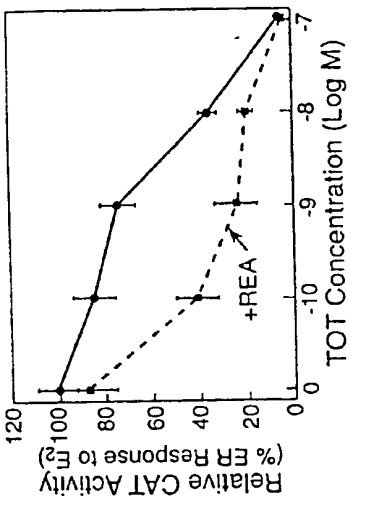


FIG. 2A

FIG. 2B

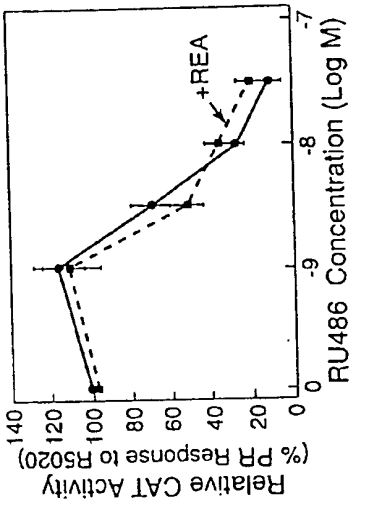


FIG. 2D

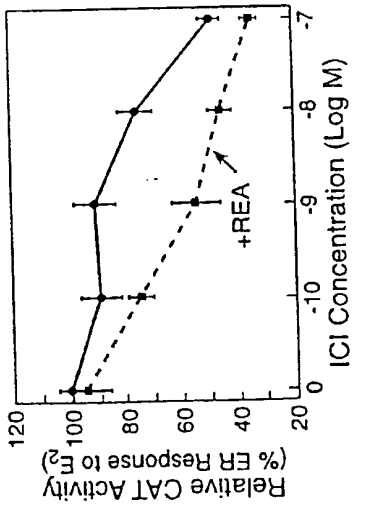


FIG. 2C

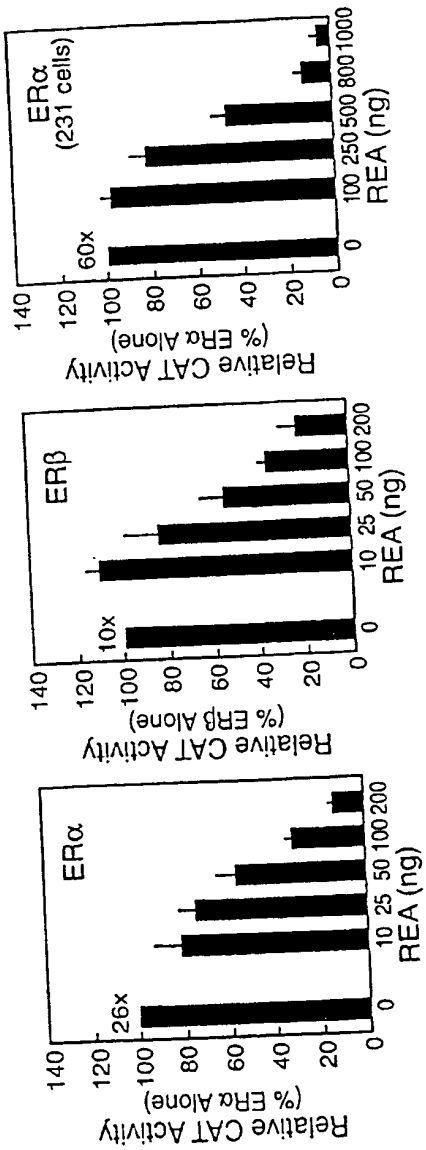


FIG. 3A

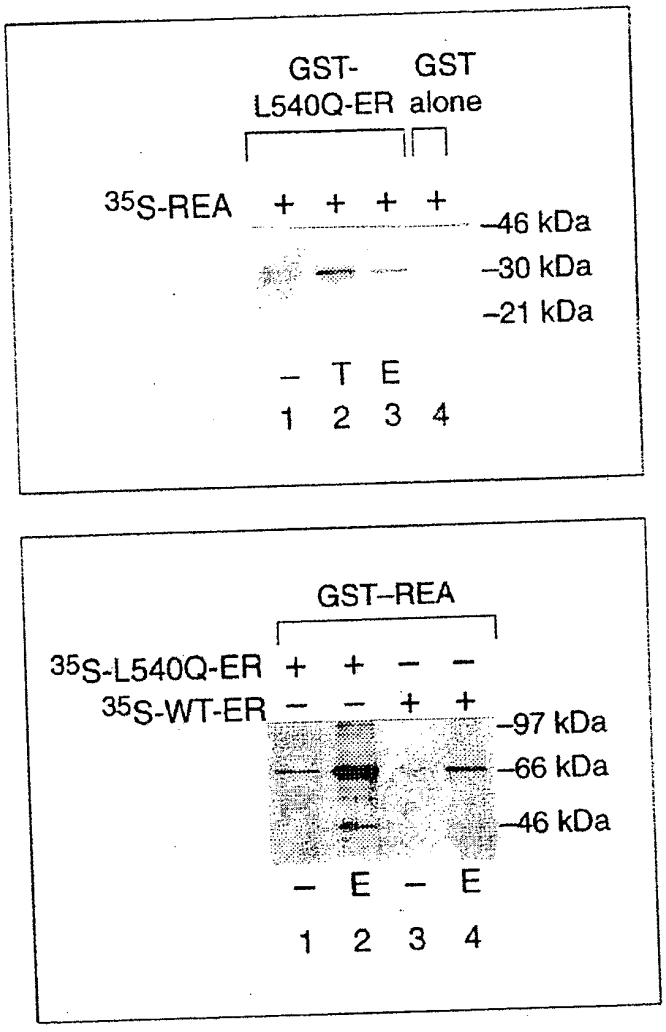
FIG. 3B

FIG. 3C

FIG. 3D

FIG. 3E

FIG. 3F


GST-
L540Q-ER
GST
alone
35S-REA + + + +
–46 kDa
–30 kDa
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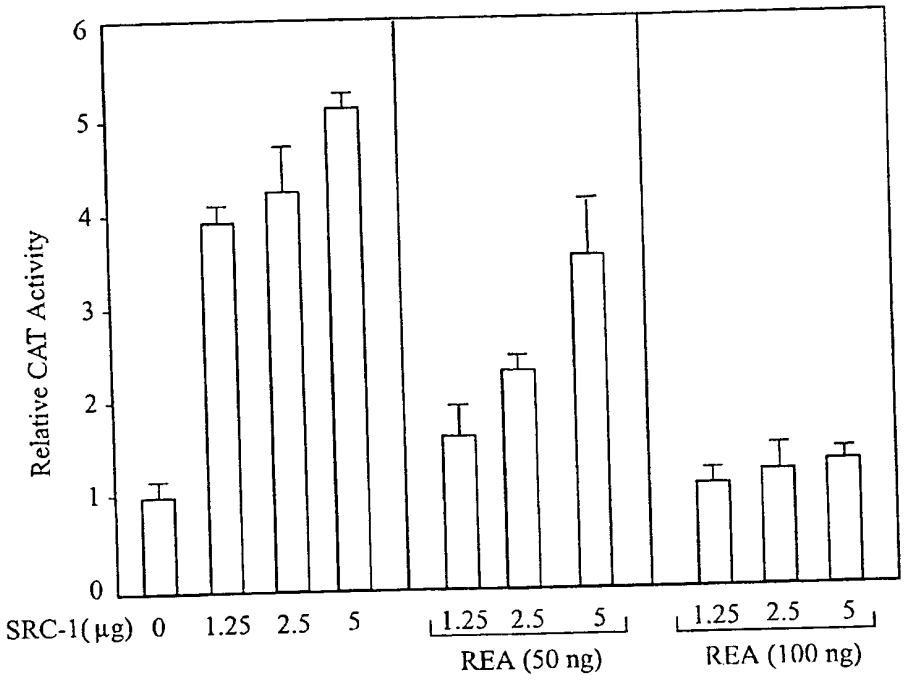
– T E
1 2 3 4
GST–REA
35S-L540Q-ER + + – –
35S-WT-ER – – + +
–97 kDa
–66 kDa
–46 kDa
– E – E
1 2 3 4

FIG. 4


Relative CAT Activity
0
1
2
3
4
5
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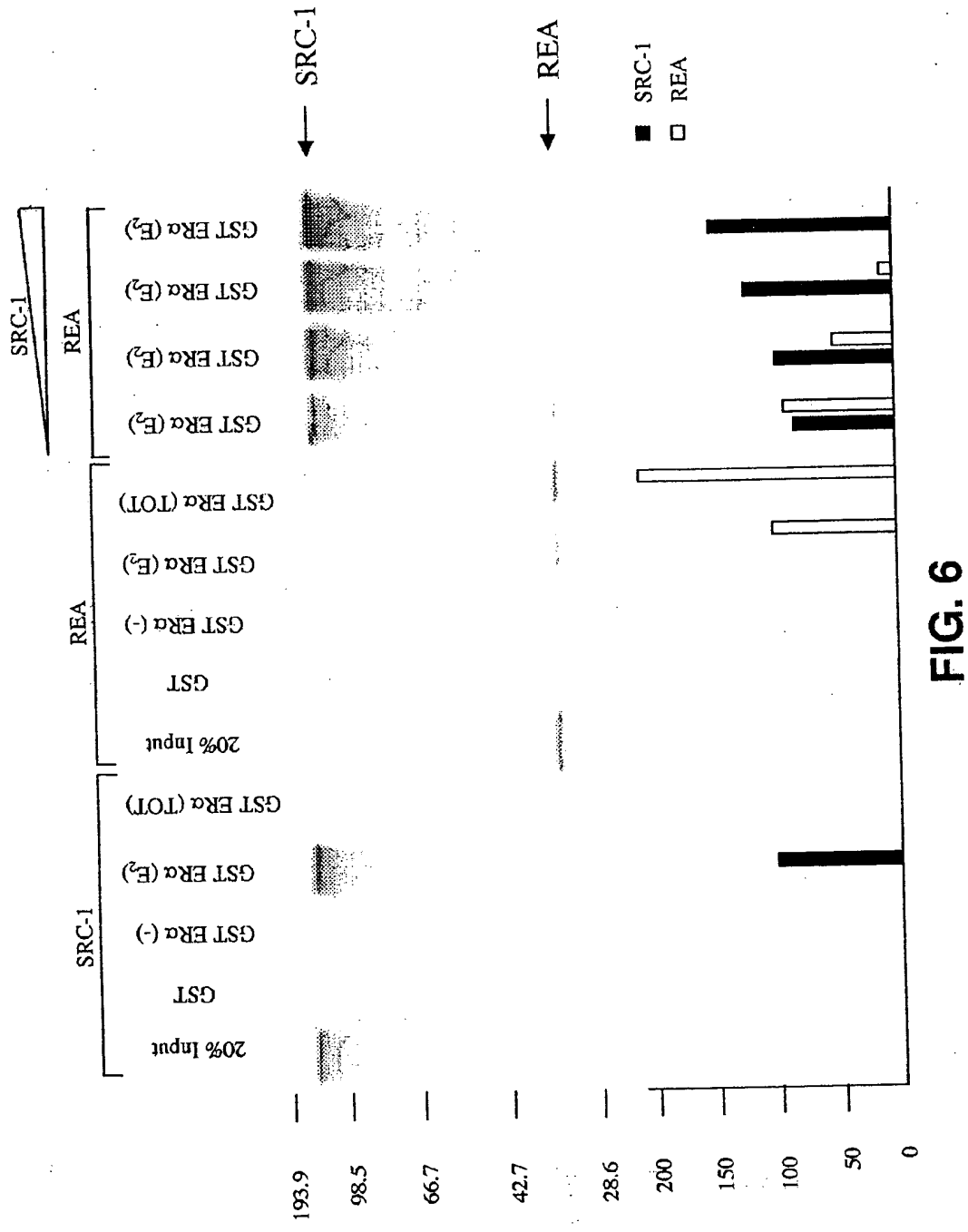
REA (50 ng)
REA (100 ng)

FIG. 5


SRC-1
REA
SRC-1
REA
GST ERα (E2)
GST ERα (E2)
GST ERα (E2)
GST ERα (E2)
GST ERα (TOT)
GST ERα (E2)
GST ERα (-)
GST
20% Input
GST ERα (TOT)
GST ERα (E2)
GST ERα (-)
GST
20% Input
193.9
98.5
66.7
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28.6
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0
SRC-1
REA
SRC-1
REA

FIG. 6

SEQUENCE LISTING

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ctcaagagtg tggtggccaa gttcaatgcc tcacagctga tcacccagcg ggcccaggta 480
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35 40 45

Ala Ile Phe Phe Asn Arg Ile Gly Gly Val Gln Gln Asp Thr Ile Leu
50 55 60

Ala Glu Gly Leu His Phe Arg Ile Pro Trp Phe Gln Tyr Pro Ile Ile
65 70 75 80

Tyr Asp Ile Arg Ala Arg Pro Arg Lys Ile Ser Ser Pro Thr Gly Ser
85 90 95

Lys Asp Leu Gln Met Val Asn Ile Ser Leu Arg Val Leu Ser Arg Pro
100 105 110

Asn Ala Gln Glu Leu Pro Ser Met Tyr Gln Arg Leu Gly Leu Asp Tyr
115 120 125

Glu Glu Arg Val Leu Pro Ser Ile Val Asn Glu Val Leu Lys Ser Val
130 135 140

Val Ala Lys Phe Asn Ala Ser Gln Leu Ile Thr Gln Arg Ala Gln Val
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Ser Leu Leu Ile Arg Arg Glu Leu Thr Glu Arg Ala Lys Asp Phe Ser
165 170 175

Leu Ile Leu Asp Asp Val Ala Ile Thr Glu Leu Ser Phe Ser Arg Glu
180 185 190

Tyr Thr Ala Ala Val Glu Ala Lys Gln Val Ala Gln Gln Glu Ala Gln
195 200 205

Arg Ala Gln Phe Leu Val Glu Lys Ala Lys Gln Glu Gln Arg Gln Lys
210 215 220

Ile Val Gln Ala Glu Gly Glu Ala Glu Ala Ala Lys Met Leu Gly Glu
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Ala Gln Asn Ile Ser Lys Thr Ile Ala Thr Ser Gln Asn Arg Ile Tyr
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Leu Thr Ala Asp Asn Leu Val Leu Asn Leu Gln Asp Glu Ser Phe Thr
275 280 285

Arg Gly Ser Asp Ser Leu Ile Lys Gly Lys Lys
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26233

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 1/00
US CL : 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/130.1, 198.1, 277.1; 435/320.1; 514/44; 530/350, 399, 402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	TERASHIMA, M. et al. The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. EMBO Journal. 1994, Vol. 13. No. 16, pages 3782-3792, especially Fig 6.	1-4,25,36 ---------- 5-7,23-25-36
Y	ZHANG et al. A nuclear receptro corepressor modulates transcriptional activity of antagonist-occupied steroid hormone receptor. Molecular Endorinology. 1998, Vol. 12, No. 4, pages 513-524, see entire document.	1,7,20,28
Y	US 5,639,616 A (LIAO et al.) 17 June 1997 (17.06.1997), see entire document.	5,7-13, 23,24,26,28,29,31
Y	SMITH et al. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifin. Molecular Endocrinology. 1997, Vol. 11, No. 6, pages 657-666, Fig. 5,6, see entire document.	1,7,20,28
Y	JANKNECHT et al. Rapid and efficient purification of native histidine-tagged proteins expressed by recombinant vaccinia virus. Proceedings of the National Academy of Sciences 1991, Vol. 88, pages 8972-8976, see entire document.	5,6,27
Y Y	SHEEHAN, C. Chapter 14: Labeled immunoassays. In Clinical immunlogy; principles and practice. Lippencott-Raven Pub. 1997, 2nd Ed. pages 145-160, see entire document.	5,7,20,26,28 8-17,19-22

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 26 January 2000 (26.01.2000)	Date of mailing of the international search report 07 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Ulrike Winkler, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26233

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHESKIS et al. Modulation of nuclear receptors interactions by ligands: Kinetic analysis using surface plasmon resonance. Biochemistry. 1996, Vol. 35, No. 10, pages 3309-3318, see the document.	18

INTERNATIONAL SEARCH REPORT

International application No.
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Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim Nos.: 4,25 and 36
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims were not searched because the sequences were not entered into the data base.

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/26233

Continuation of B. FIELDS SEARCHED Item 3: West, Medline, Biosis
repressor, estrogen receptor, antiestrogen, agonist, steroid hormone receptor, GST-fusion protein, His-tagged protein, ELISA